Leafhoppers as indicators for risk assessment of GM biofortified crops

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Abstract: As striking advances have been made in the last years to produce a range of biofortified GM crops with an increased level of nutrients, new approaches for environmental risk assessment on non-target arthropods have to be developed. In particular we focus on a new multivitamin maize developed at our university (Naqvi et al., 2009), producing an increased level of beta carotene, ascorbate and folate. We argue that problem formulation becomes extremely complex for this GM maize both at the plant and arthropod level. First, although the functions of carotenes and other vitamins are relatively well studied in plants, little is known about how biofortified plants modulate the metabolic pathways to increase the production of these compounds and which are their associated trade-offs. Second, studies on vitamins in insect systems are scarce, especially on their movement among trophic levels.
We propose *Zyginidia scutellaris* (Auchenorryncha: Cicadellidae) as an indicator species to assess risks of GM maize to non-target herbivores guided by the use of the best predicted power versus replication relationships from previous field trials. Additionally, we hypothesize that this species is the base to build an indicator maize trophic chain given that it is the most abundant herbivore in maize fields. To explore the suitability of leafhoppers as indicators we present a literature review on the effects of insect resistant and herbicide tolerant GM crops and non-GM varieties on different leafhopper species. Finally we suggest an ecological risk assessment as the only way to detect the potential cascading effects of multivitamin crops.

Key words: cicadellidae, biofortified maize, multivitamin maize, risk assessment, problem formulation

1. Introduction

New generations of GM crops are being developed globally. Many of these new generation crops imply generally a modified metabolism of the plant, as the recent advances in genomics have allowed to target new genes of tolerance to biotic stresses (e.g. involving lectins, RNAi, etc) abiotic stress (e.g. tolerant to drought, salt, heat, and future “climate ready” crops), and to engineer other crops with modified metabolism that confer desired attributes to the plant, like biofortified crops. The scientific principles underlying the environmental risk assessment for non-target arthropods (NTAs), completed for herbicide-tolerant and insect-protected GM crops commercialized to date, need now to be applied to these new biofortified crops.

In this paper we deal in the first place with vitamin biofortified crops, and we explore the basis for the current regulatory frameworks in the potential countries of adoption, mainly the African continent. Secondly we study the case of GM Multivitamin Maize (MVM), present the potential changes in the MVM that may have occurred due to the genetic modification. In the third place we introduce the leafhopper *Zyginidia scutellaris* (Hemiptera: Cicadellidae as the candidate indicator species of GM maize impacts on the maize food web; and propose an NTA Environmental Risk Assessment for MVM that follows a tiered approach through trophic relationships.

2. Regulatory systems: Europe, US and Africa

Regulatory frameworks governing GM crops vary widely throughout the world, but essentially they are either developed specifically for GM crops, or they are adapted from existing legal instruments that apply to conventional agriculture (Ramessar et al., 2008). In the EU there is a process-based approach for the regulation of GMOs as the breeding techniques used for their production are considered new and raise specific safety concerns and thus a specific legislation was developed. The actual directive 2001/18/EC on the release of GMOs into the environment stresses the need for a common methodology for Environmental Risk Assessment (ERA), and broadens the risk assessment criteria from the older directive to include direct, indirect, immediate, delayed and cumulative long-term adverse effects and establishes an obligatory Post Market Environmental Monitoring.

In contrast, in the US there is a product-based approach to regulate GMOs, where the legislation focuses on the risks of the products and not the breeding techniques. Thus, GM plants and products are regulated by the existing regulatory system. Most developed countries have introduced regulations that share features of both the EU and US systems, the regulation of GM crops worldwide has been reviewed by Ramessar et al. (2008) and Paoletti et al. (2008).

The adoption of GMOs in developing countries and particularly in Africa has been strongly influenced by developed countries, and particularly the EU and USA. In fact, there is the opinion that the polarized debate about GM crops and their regulation has been an obstacle for the adoption of this new technology in Africa (Adenle, 2011; Paarlberg, 2010). In contrast, GM crop biofortification has been developed to reach malnourished rural populations in the African continent and deliver micronutrients, like minerals and vitamins, that may alleviate chronic diseases. At the present time there are still some African countries with no biotechnology regulatory systems (e.g. Angola, Chad and Somalia) while others have established legal instruments that enable them to regulate GMOs to varying extents (e.g. Burkina Faso, Egypt, Ghana, Kenya, Mali, Namibia, Nigeria, South Africa, Uganda, and Zimbabwe). So far, only three countries, South Africa, Burkina Faso, and Egypt, have commercialized GM crops, while a few others have or are conducting confined field trials (AU-NEPAD African Biosafety Network of Expertise, 2011 www.nepadbiosafety.net).

In all regulatory systems comparative risk assessment is a fundamental principle of GM plant ERA, and it is mostly based on the concept of substantial equivalence. The principle of substantial equivalence stipulates that new GM varieties should be assessed for their safety by comparing them with an equivalent, conventionally bred varieties that have an established history of safe use (Codex, 2003, EFSA, 2011). GM crop lines have to be screened for phenotypic and compositional equivalence in order to confirm or falsify the risk hypothesis that the GM crop is not different from the non-GM crop other than the presence of the introduced gene(s), the expression of the gene(s), and the intended phenotype (Nickson, 2008). Thus, the biologically meaningful differences observed between the GM plants and its comparators are an outcome of the genetic modification (Wolt et al., 2010) and are the ones to evaluate when developing an ERA for NTAs.

3. Emerging biofortified crops

Biofortification aims to reach malnourished rural populations who may have limited access to a diverse diet, dietary supplements and commercially fortified foods. The most popular traits used for plant biofortification are high mineral and vitamin density (Beyer, 2010). As the Table 1 reflects there is a particular interest in breeding crops containing provitamin A or carotenes and iron, both through transgenesis and conventional breeding.

Some of these biofortified crops obtained through programs of conventional breeding are already cultivated and others obtained by transgenesis are on the pipeline, including the famous Golden Rice II. Conventionally bred provitamin A maize varieties were released in Zambia (three varieties) and Nigeria (two varieties) in 2012 (Saltzman et al., 2013). As for Golden Rice II, two seasons of multi-location field trials have been completed in The Philippines (for details http://www.philrice.gov.ph/?page=golden) and data from these trials must next be submitted to Philippine government regulators for their evaluation as part of the biosafety approval process.

Table 1. Summary of the provitamin A and iron biofortified crops that are developed or under development, and the country and year of their past or expected deployment (adapted from Saltzman et al., 2013)

	Conventional breeding			**Genetically modified**		
Nutrient	Crop	Country	Release	Crop	Country	Release
Provitamin A/ Carotenoids	Banana	Nigeria Ivory Coast Cameroon Burundi DR Congo	?	Rice*	Philippines Bangladesh Indonesia India	2014?
				Sorghum	Kenya Burkina Faso Nigeria	2018 all countries
	Cassava	DR Congo Nigeria Brasil	2008 2011 2009			
	Maize	Zambia Nigeria Brazil China India	2012 2012 2013 2015 ?			
	Pumpkin	Brazil	2015			
	Sweet potato	Uganda Mozambique Brazil China	2007 2002 2009 2010			

Provitamin A/ Carotenoids + Iron	Banana Cassava	Uganda Nigeria Kenia	2019 2017 both

*Golden Rice II

Curiously though Golden Rice has been a flagship biotech crop for the last 10 years, to our knowledge, no scientific (public) literature on potential impacts on NTA is available. So, either an ERA has not been developed yet or it is for developers and regulators eyes only. It has been argued that when the introduced gene has no reasonable mechanism for conferring toxicity to organisms, like in the case of biofortified crops, it is unlikely that detailed knowledge of the mechanism by which a gene confers the desired properties will be necessary for the risk assessment (Nickson, 2008). Still, experience shows that unintended effects might still take place, and with the existing GMO regulation in Europe a sound ERA for biofortified crops has to be developed. Both scientists and regulators appeal to establish which is the basis for comparability and the parameters to identify "meaningful changes" in the transformed plant as to date no limits of concern have been set (Wolt et al., 2010).

4. The plant: Biofortified multivitamin maize

The Applied Plant Biotechnology Laboratory at UdL created an elite inbred South African transgenic maize plant in which the levels of 3 vitamins were increased specifically in the endosperm through the simultaneous modification of 3 separate metabolic pathways (Naqvi et al., 2009). The kernels of this multivitamin maize contain 169-fold the normal amount of beta-carotene (provitamin A), 6-fold the normal amount of ascorbate (vitamin C), and double the normal amount of folate (vitamin B9).

The selectable marker bar and 4 genes/cDNAs encoding enzymes of the metabolic pathways for the vitamins were introduced: 1) the maize (*Zea mays*) phytoene synthase (*psy1*) cDNA under the control of the wheat LMW glutenin promoter and the *Pantoea ananatis* (formerly *Erwinia uredovora*) *crtI* gene (encoding carotene desaturase) under the control of the barley D-hordein promoter were introduced to increase beta-carotene levels; 2) the rice dehydroascorbate reductase (*dhar*) cDNA to increase ascorbate levels; 3) the *E. coli folE* gene encoding GTP cyclohydrolase (GCH1) under the control of the barley D-hordein promoter to increase folate levels.

European ERAs requires a thorough evaluation of environmental effects of crops obtained through transgenesis by exploring the possible scenarios of harm. As the traits introduced to maize do not have toxic properties, the potential impacts on the arthropod maize community will mainly derive either from a diet enrichment for herbivores or from unintended changes in the plant. To explore these impacts we summarize the potential plant changes that MVM may have experienced and its potential implications for NTA.

5. Potential plant changes produced by the insertion of the 3 metabolic pathways and implications for NTAs

In order to develop a sound ERA for NTAs for the case of MVM we have to define and identify potential differences in the plant that may plausibly lead to an impact to the herbivore community and the subsequent trophic levels. To our understanding the potential changes between the MVM and its isogenic counterpart may be due either to the intended effects, i.e., a vitamin overexpression in the endosperm, or unintended effects that may take place throughout the plant, consequence of the changes of metabolic pathways in the endosperm or other cascading effects derived from the gene insertion, regulation or interaction of products.

1) Vitamin overexpression in the endosperm

The overexpression of vitamins in MVM variety is not constitutive as the 3 metabolic pathways have been engineered with endosperm specific promoters and thus we expect the vitamin overexpression to be tissue specific. In fact, Diretto et al. (2007) obtained a GM carotenoid rich potato achieved both under constitutive and tuber-specific overexpression of a bacterial pathway. In this work the authors found that the constitutive expression of the *crtY* and/or *crtI* (the same as MVM) genes interferes with the accumulation of leaf carotenoids, but that the expression of the genes under tuber specific promoter control results in tubers with a ‘‘golden’’ phenotype without any adverse leaf phenotypes. Consequently we expect that the accumulation of vitamins in MVM takes place in maize kernels and that affects predominantly insects that feed directly on the maize cob. A good surrogate to test the effect of a vitamin rich food on insects in our conditions might be the secondary pest *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae).

Although the function of carotenes and ascorbate is relatively well studied in plants (Asensi-Fabado & Munné-Bosch, 2010; DellaPenna & Pogson, 2006), studies on plant-derived vitamins in insect systems are scarce. Carotenes and ascorbate act as antioxidants in living organisms but in addition they fulfil other physiological and ecological roles. Two recent papers review carotene and ascorbate function in plants and insects and give insight to the complex plant-insect and insect-insect interactions mediated by these vitamins (Goggin et al., 2010; Heath et al., 2012); a summary of the functions of carotenes and ascorbate in plants and insects is summarized below (Tables 2 and 3).

Multiple hypothesis can be derived from the literature. The most straightforward would be that if an insect species had a shortage of any of these vitamins in its diet, these multivitamin cobs may produce fitter insects (e.g. reinforce their immune response) than the ones feeding on conventional maize. This fitter insects may be able to live longer or/and produce more offspring, and might result in a population explosion of the pest. In southern Africa *Chilo partellus* (Pyralidae: Lepidoptera) and *H. armigera* may be the species provided with an extra vitamin content if MVM was to be cultivated, and thus become focal pest species for risk assessment.

Table 2. Carotenoid synthesis and function in plants and insects (based on Heath et al., 2013).

Plants	Insects
Synthesized in plastids	*Most insects cannot synthesize them (exceptions related to fungus gene transfer: Acyrthosiphon pisum, Bemisia tabaci, Tetranychus urticae)**
Harvests light energy during photosynthesis	Is involved in coloration, vision, diapause and photoperiodism
Quenches Reactive Oxygen Species produced during photosynthesis and plant stress	Serves as antioxidants (UV radiation/oxidative stress)– Immune response
Is a precursor of signaling molecules that influence development and biotic/abiotic stress responses	
Precursor of semiochemicals	Is a precursor of pheromones and mediates interspecific interactions

*(Moran & Jarvik, 2010; Altincicek et al., 2011; Sloan & Moran, 2012

Table 3. Ascorbate (vitamin C) synthesis and functions in plants and insects (based on Goggin et al., 2010)

Plants	Insects
Synthesized in mitochondria	*Not clear whether insects can synthesize it.*
Controls gene expression and cell growth	Controls molting process
Quenches Reactive Oxygen Species produced during photosynthesis and plant stress	Modulates humoral and cellular immune responses.
	Regulates accumulation of energy reserves in the haemolymph.
Is a signaling molecule involved in plant response to plant stress	Detoxifies plant allelochemicals
Participates in the regeneration of VitE and in the synthesis of organic acids	
Is involved in phythormone and flavonoid biosynthesis and in the xanthophyll cycle	

2) Unintended effects

Unintended effects are difficult to hypothesize and may be sometimes rather speculative. In the case of MVM, we have identified two possible unintended effects: (1) Physiological trade-offs as a consequence of vitamin overexpression; and (2) Other unintended effects derived from the transgenesis.

a) Physiological trade-offs as a consequence of vitamin overexpression

The precursors of the vitamin synthesis are the sugar pools in the cell. These assimilates are produced in the green tissues of the plantduring the process of photosynthesis and translocated through the phloem to the sink organs. Our hypothesis is that, in the case of MVM, the endosperm and its vitamin production pathways might act as a stronger sink as more assimilates are needed to produce more vitamins. The alterations of the metabolic fluxes toward these vitamin production pathways might affect the availability of intermediates for correlated pathways, or limit the amount of assimilates in other tissues of the plant, with relevant consequences for plant development and fitness. Nevertheless, the relationships between carbohydrate availability and secondary compounds synthesis cam be extremely complex and difficult to decode (Fanciullino et al., 2013).

On the other hand as vitamins are part of the plant secondary metabolism probably the impact of the "stronger sink" will not be as relevant as for other modified metabolism crops involved in the primary metabolism of the plant or that express genes of the secondary metabolism constitutively. It remains to be seen whether this overproduction of vitamins in MVM is really free of endogenous regulation.

b) Other unintended differences

Unintended differences in transgenic and non-GM plants can be predictable or unpredictable as a function of whether they are expected and explicable in terms of the present knowledge of plant metabolism and physiology or whether they fall outside our present level of understanding (Cellini et al., 2004). Unintended effects may occur as a consequence of (1) pleiotropic effects of the integrated DNA on the host plant genome as a result of transgene products interacting with the regulation of other genes or the activity of other proteins (transgene specific) (2) host gene disruption or DNA sequence rearrangements at the insertion site (event specific) (3) host plant genome modification by the process to obtain GM plants.

Both targeted and untargeted approaches can be used to explore unintended effects. Current risk assessment of GM maize includes a targeted analysis of nutrients, anti-nutrients, allergens and secondary metabolites identified by an OECD consensus document (OECD, 2002) as the key compounds for maize, using validated analytical methods.

Targeted approaches have been able to detect unintended differences in GM maize. For example (Saxena & Totzky, 2001) detected higher lignin levels in insect resistant transgenic maize stems than in conventional isogenic lines, and (Poerschmann, et al., 2005) observed differences in lignin composition. It has been suggested that untargeted profiling techniques at different biological levels (transcripts, proteins and metabolites) may be the future to screen any of the potential unexpected differences among GM and conventional lines (Cellini et al., 2004; Ricroch et al., 2011). Using transcriptome, proteome and metabolome profiling, Barros et al. (2010) found that the environment (plants were grown over three seasons in one location) affected more strongly gene expression, protein distribution, and metabolite content of kernels of two GE maize lines (MON810 and glyphosate tolerant) than the genetic modification. The main drawback is that both approaches and most studies usually target GM food and feed safety issues, and consequently the unintended changes in the GM plant as a whole are not explored further than at the phenotype level.

One of the most well-known unintended effect of GM crops on the arthropod food web is the case of higher abundance of homopterans in Bt maize. Lumbierres et al. (2004) and Pons et al. (2005) found a significantly higher rate of offspring production by colonizing alate mothers of *Rhopalosiphum padi* (Hemiptera: Aphidae) and consequently higher densities of this species on Bt maize. These unexplained differences between GM crops and its comparators may scale up to the following trophic levels, as it was reported by Faria et al. (2007). The authors observed a positive effect of Bt maize on the performance of the aphid *Rhopalosiphum maidis* that led to an enhanced the performance of parasitic wasps that feed on aphid honeydew. They also showed that two of the three transgenic/isogenic plant pairs studied differed significantly in the amino acid concentrations of the phloem sap.

We believe that the MVM and its isogenic counterpart provide a case study in which, apart from the endosperm, we can compare effects of plants with few genetic differences (3 genes + promoters-metabolic pathways) on insects, and as a consequence the potential unintended effects of the breeding technique in non-targeted tissues may be inferred.

6. The leafhopper *Z. scutellaris* as an indicator of impacts of GM maize

Zyginidia scutellaris is a widely distributed species in Europe and is considered a secondary pest of maize in Spain, France and Germany, though it is rarely of economic importance. It is an oliphagous feeder on Poaceae and it may build up high density populations during summer in the maize. As a mesophyll feeder, the species causes damages in by producing pale stripes on the leaves, with a preference for the older ones. This leafhopper species has been recorded for years in maize field trials in Spain (Eizaguirre et al., 2006; Pons et al., 2005) and in Germany (Rauchen et al., 2008, 2010).

Why do we choose the maize leafhopper as an indicator species? First of all for its relevance; population densities of *Z. scutellaris* in maize are often high and can exceed those of other herbivores (Pons et al., 2005, Albajes et al., 2009, 2011). They perform an important functional role as herbivores in maize arthropod communities and their populations have been reported to be the base of an indicator food web (Albajes et al., 2011).

Secondly, this leafhopper shows high statistical power in field trials, in fact it is the taxon with the best detectability both in meta-analysis and single field trials (Comas et al., 2013). Statistical power, which represents the probability that an incorrect null hypothesis will be correctly rejected by a particular test, has been suggested to be an important criterion for selecting indicator species and it can indicate the quality of sampling in a way that addresses the adequacy of experimental designs (Prasifka et al., 2008).

In the third place, homopterans are insects with a high potential sensitivity to plant quality and environmental changes. It has been seen that selected aphid species prefer and perform better in some genotypes or in plants that differ in quality (Mooney, Pratt, & Singer, 2012; Powell, Tosh, & Hardie, 2006; Zytynska & Preziosi, 2011). Less information is available for leafhoppers but we think they might behave similarly. For example a recent article reported that *Empoasca* leafhoppers are able to identify jasmonate mutants in natural populations of *Nicotiana attenuata* (Kallenbach et al., 2012).

To explore the topic of leafhopper performance on transgenic crops we did a literature compilation of laboratory and field studies that tested leafhoppers on transgenic crops and their isogenic counterparts. We performed this search in the Scopus database using the keywords: cicadellidae, GM crop, Bt, Ht. From the output of the search we selected those published studies that were dealing with taxonomically determined leafhopper species and withdrew those studies that evaluated the "Cicadellidae" all together. Also we selected those studies that were "clear" in their choice of GM varieties (mentioned the variety name and the trait/s introduced) and in their methods and results. We selected 4 laboratory studies (Table 4) and 10 field studies (Table 5).

Table 4. Published laboratory studies testing the effects of a GM crop on leafhoppers.

Crop	Varieties	Stressor	Species	Parameters	Effect*	Country	Reference
Maize	Bt(Event 176)	Cry1Ab	*Zyginidia scutellaris*	Bt content on predator	+	Spain	(Obrist et al., 2006)
	Bt germplasm/ isogenic	Cry1F	*Dalbulus maidis*	Oviposition Egg haching rate	+ -	Argentina	(Virla et al., 2010)
Rice	Lectin transgenic/isogenic	GNA	*Nephotettix virescens*	Mortality Feeding preference GNA on honeydew	+ + n.d.	UK	(Foissac et al., 2000)
	Lectin transgenic/isogenic	ASAL + GNA	*Nephotettix virescens*	Mortality Development Fecundity Feeding activity	+ - - -	India	(Bharathi et al., 2011)

*** + The effect detected for the GM plant is higher/faster than in the isogenic counterpart**
- The effect detected for the GM plant is lower/slower than in the isogenic counterpart
n.d. Not detected

From the laboratory studies we can conclude that the parameters mortality, development and fecundity and plant choice have been able to detect differences between the GM crop and its isogenic counterpart. Though the number of laboratory studies is limited, and two of the studies focus on GM rice varieties designed to control homopteran pests, we believe that the above mentioned life history traits should be assessed when considering the potential effects of MVM on *Z. scutellaris*.

In contrast, field studies focus mostly on arthropod abundance. Results show that differences in the abundance of leafhoppers between the GM varieties and their isogenic counterparts (and treatments in some case, e.g. Ht maize) can be detected, but multi-year studies show that these differences are depending on the year and probably also on the method used.

Table 5. Published field studies testing the effects of a GM crop on leafhoppers.

Crop	Varieties	Stressor	Species	Parameters	Effect*	Country	Reference
Maize	Bt (MON810)/ isogenic	Cry1Ab	*Zyginidia scutellaris*	Abundance (visual) Damage (SPAD)	+ 0	Spain	(Pons et al., 2005)
	Ht/ isogenic plus herbicide regime	Ht + management	*Zyginidia scutellaris*	Abundance (visual)	+/0 (year dependent)	Spain	(Albajes et al., 2009, 2011)
	Bt (Event 176)	Cry1Ab	*Zyginidia scutellaris*	Bt content (ELISA)	+	Spain	(Obrist et al., 2006)
	Bt (MON810)/ isogenic	Cry1Ab	*Zyginidia scutellaris*	Abundance (visual, sweep netting, yellow traps, custom made sticky traps)	+/0 (year/method dependent)	Germany	(Rauschen et al., 2008)
	Bt (event MON88017)/ isogenic	Cry3Bb1	*Zyginidia scutellaris*	Abundance (sweep netting, custom made sticky traps)	+/0 (year dependent)	Germany	(Rauschen et al., 2011)
	Bt (Herculex Elite)/ isogenic	Cry1F	*Dalbulus maidis*	Abundance (visual)	+	Argentina	(Virla et al., 2010)
Potato	2 Newleaf/ isogenic	Cry3a	*Empoasca fabae*	Abundance (sweep netting, visual) Damage (visual %)	0 0	USA	(Kaplan & Dively, 2008)
	Bt (Newleaf)	Cry3a	*Empoasca fabae*	Abundance (meta-analysis)	+/0	Canada	(Cloutier et al., 2008)
Rice	Bt (TT9-3)/ isogenic	Cry1Ab+Cry1Ac	*Nephotettix cincticeps* *Thaia subrufa* *Recilia dorsalis*	Composition Abundance (yellow sticky traps, Malaise traps, vacuum-suction)	0 0	China	(Chen et al., 2006)
Cotton	3 Bt/3 isogenic	Cry1Ac	*Amrasca biguttula*	Abundance (visual)	+/0 (year dependent)	India	(Sharma & Pampapathy, 2006)

*** + The effect detected for the GM plant is higher/faster than in the isogenic counterpart**

- The effect detected for the GM plant is lower/slower than in the isogenic counterpart

0 No differences detected

6. The leafhopper *Z. scutellaris* as an indicator of impacts of multivitamin maize on non-target organisms

In summary, we can say that leafhoppers, and in particular the maize leafhopper, might be a good species to evaluate the impact of transgenic plants on the arthropod food web. Following a recent case study concerning the development of risk hypotheses for invertebrates exposed to a GM ryegrass with elevated triacylglyceride levels (Barratt et al., 2011) we developed the change hypotheses for *Z. scutellaris* feeding on MVM and the maize food-web. The process employs a stepwise analysis of the trophic relationships within the community following the tiered-approach recommended for ERA of NTAs (Romeis et al., 2008). Again, for MVM hypotheses are not as explicit as in the above mentioned study on GM rygrass, where the higher density of lipids in the plant is a constitutive trait, and consequently it is the main factor to cause changes in the insect community. For this motive we think that an ecological approach for the risk assessment of NTAs is the only way to detect the potential cascading effects of multivitamin crops, especially in the scope of the current GM regulation in the EU.

The nature of the trait introduced into the GM crop greatly influences the kind of risk assessment studies that need to be conducted to effectively evaluate these novel crops. An example of this is a recent paper that compares the regulation of GM crops containing dsRNA between three countries and suggests improvements to be made in risk assessments (Heinemann et al., 2013). In all GM crops unintended effects may occur. One big challenge for regulatory systems will be to establish which differences are considered "acceptable" differences between a GM crop and its isogenic counterpart (Nickson, 2008; Wolt et al., 2010), and to consider at the same time the nature of all new generation GM crops.

Table 5. Hypothesized changes in *Zyginidia scutellaris* life history traits, and on the maize food web, when feeding on MVM (following Barratt et al., 2011).

Level 1. *Zyginidia scutellaris* feeding on MVM exhibit the following changes:	
Physiological	Improved survival Nymphs grow more rapidly Nymphs and adults have higher biomass Adult females have higher fecundity
Phenological	More generations per year
Behavioral	Larger individuals consume more vegetation
Level 2. Some Level 1 effects are demonstrated, so consider:	
Population effect	Species has increased fitness, density and competitive ability, stronger immune system/reserves

Tritrophic effect	Natural enemies benefit by changes in host fitness and phenology
Effect on vegetation	MVM under increased pressure from herbivores
Level 3. Some Level 2 effects are demonstrated, so consider:	
Trophic cascade effect	Other prey/hosts at increased risk from fitter natural enemies
	Reduced impact on plants from other herbivores which are under an increased NE pressure

7. Implications for Environmental Risk Assessment

Formulation of risk hypotheses is extremely challenging for insects feeding on multivitamin maize plants and other crops with modified metabolism as little is known about how these plants modulate the metabolic pathways to increase the production of these compounds and which are their associated trade-offs. Problem formulation is complex also in the scope of current regulatory frameworks as we have to define what to protect from harm when we no longer deal with a transgene that produces toxic compounds, or that may affect directly other organisms by its associated practices (e.g. herbicide tolerant crops, and herbicide applications that may cause flora changes).

Due to the above mentioned reasons we propose that the ERA of NTAs of biofortified crops should focus on species that (1) feed on tissues that accumulate the biofortified elements, e.g. *H. armigera* in the case of MVM as it feeds on the cob that overexpresses the 3 metabolic pathways; (2) are key players in the crop's food web (representive of trophic levels), and if possible that have proved statistical power. The leafhopper *Z. scutellaris* meets these last requirements and is thus a suitable indicator herbivore to detect unintended effects of MVM on arthropods. For this, ERA for MVM on NTA should focus in traits related to the physiology, phenology and behavior of the leafhopper to hypothesize which effects might be expected on the maize food web. With our selected system MVM-*Z. scutellaris* we will be able to test whether our indicator species is sensitive enough to detect small nutritional changes in plant tissues.

In the near future, regulatory frameworks will have to adapt to GM crops with enhanced nutritional traits (and other "new generation" traits) and probably we will see how the established ERA, inherited from toxicological analysis, is revisited.

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